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A folic acid-based functionalized surface for biosensor systems

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Abstract The performance of a biosensor depends largely on its interface with the biological system. This interface imparts a biologically relevant function to the device and provides a measure of specificity towards the biological analyte of interest. This paper documents the choice of folic acid as the functional component of a cantilever sensor to recognize nasopharyngeal (KB) cancer cells. A conjugation chemistry protocol has been outlined to deploy folic acid onto a titanium-coated sensor surface using a silane linker. The presence and biological activity of the sensor was verified by means of an immmunospecific (ELISA) procedure. The overall performance of the folic acid-based cantilever sensor was measured using cancerous KB cell-binding experiments.

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1 Introduction

The etiological basis of diseases such as cancer can be traced down to genetic malfunctions and their consequent proteomic, tissue and organic manifestation. This molecular interpretation of disease provides the very basis for detection, prognosis and potential strategies for intervention at the molecular level. These strategies influence a large portion of the effort in biological nanotechnology (or bionanotechnology), directing the development of new techniques for sensing, management and possible treatment of disease.

Micro Electro Mechanical Systems (MEMS) provide a versatile platform for application in biological systems (BioMEMS). Such applications include in-dwelling sensors and monitors, therapeutic devices, microsurgery tools and *ex vivo* automated diagnostic and prognostic systems. In order to provide 'intelligence' to a BioMEMS device in the form of disease specificity, it must have a functional component capable of distinguishing between the analyte of interest among other factors in the test sample. Effective design of the functional component is a multidisciplinary effort requiring the inputs of the disease biologist, the MEMS designer and the materials engineer to integrate the functional components onto the sensor platform.

The representation of the functional layers that constitute a biosensor are shown in Fig. 1. A silicon-based micromechanical system, the cantilever, has been used as the transducer layer in the design of this biosensor. The use of the cantilever as the sensor platform is based upon previous work by the authors [1]. The development of the recognition layer for the biosensor represents a continuation of the aforementioned project. Presented here is the rationale, construction and evaluation of the recognition layer atop the cantilever

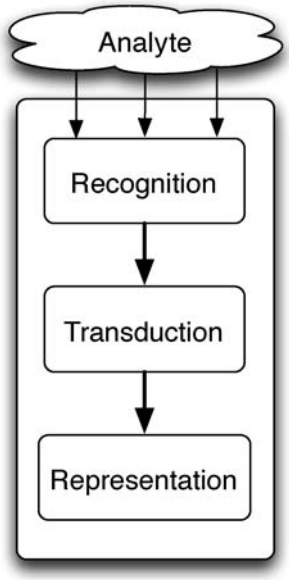

Fig. 1 The logical biosensor. A representation of the biosensor shows three logical components (or 'layers'. The recognition layer distinguishes between the analyte of interest among the other components of a sample, and makes it available for quantification. The transducer layer, which is the cantilever sensor platform in this case, converts the presence of the analyte into a machine readable signal. The representation layer converts the machine readable signal into a human-interpretable form

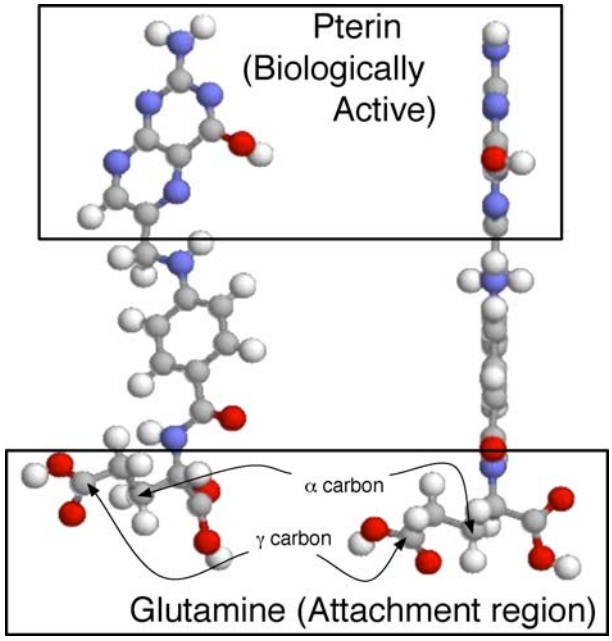

Fig. 2 The *ab initio* model of the structure of folic acid. The folic acid molecule comprises of 3 distinct moieties, the 6-methylpterin and L-glutamic acid, joined together by a p-aminobenzoic acid linkage. The pterin head is the biologically active component of folic acid and is recognized by the folate antibodies. The α-carbon in the glutamic acid end is ideal for chemical attachment of folic acid to other molecules

sensor platform in order to grant specificity towards certain cancers, notably ovarian and nasopharyngeal.

1.1 Folic acid as a recognition layer for certain cancers

Folic acid (also known as folate or pteroylglutamic acid) is a water soluble vitamin of the B-complex group. The structure of folic acid ($C_{19}H_{19}N_7O_6$) is shown in Fig. 2. Mammals are incapable of synthesizing folic acid and must obtain it from their dietary intake. The biologically active form of folic acid is called 5,6,7,8-tetrahydrofolate (THF). Reduced folates are co-enzymes in a number of biochemical pathways involving the transport of 1-carbon units like methyl ($-CH_3$), methylene ($-CH_2-$) and formimino ($-CH=NH$). As such, they are utilized in the synthesis of the DNA bases thymine and the purines [23]. This makes folate especially important to the synthesis (S) phase of the cell cycle, when the DNA is being synthesized. In fact, the chemotherapeutic drug methotrexate is a folic acid analog and is designed to exploit this local increase in demand as the drug delivery mechanism.

The cell internalizes folic acid with the help of specialized molecules on the cell surface called the *folate receptors*. As the name suggests, these molecules are responsible for the uptake of folic acid from the blood into the inside of the cell where it becomes available for the *S* phase. The selective increase in uptake of folate by malignant cells led to the idea that folate could be used as a targeting moiety for various anti-cancer drugs. The delivery of imaging agents [24], radionucleides [17], chemotherapeutic agents [9], gene therapy vectors [3, 6, 14, 18] and other devices such as antibodies [10] has been accomplished by tagging the folate molecule to function as a selective delivery device.

There are four types of folate receptors, labeled, FR $-\alpha$, FR $-\beta$, FR $-\gamma$ and FR $-\gamma'$. The folate receptor $-\alpha$ has been seen present normally in the largest amount in the choroid plexus, lungs, kidneys and the thyroid. It has been seen in 4 out of 6 metastatic brain tumors [25]. It is also seen in malignant and benign lesions in the human female genital tract [7] and in nasopharyngeal carcinoma [8, 19]. It can also be secreted from receptor-rich cells and has been considered an important serological marker for ovarian cancer [13]. In general, FR $-\alpha$ is often over-expressed in malignancies of epithelial origin and may be present as a cell surface receptor or a soluble entity [4]. The folate receptor has a high affinity towards folic acid [$K_d \approx 0.1 - 1\,nM$] and it aids the reduced folate carrier molecule (RFC) in internalizing folic acid across the cell wall [4]. The RFC has a much smaller affinity for folic acid and it requires the services of the folate receptor to achieve high local concentration of the folic acid around itself [4]. The time scale over which the folate is internalized and the folate receptor becomes available for further attachment is not yet known.

1.2 Attachment of folic acid to the sensor surface

The structure of folic acid as determined from *ab initio* modeling is shown in Fig. 2. The 6-methylpterin end (also known as the pterin end) is recognized by the folate receptor. It is thus necessary that the attachment to the sensor surface must occur such that the pterin end is available for recognition. The attachment to the sensor surface must therefore occur through the glutamine end. Further, from steric considerations, the folic acid molecule needs to be attached through its α carbon. The hairpin bend in the pterin end will render the folic acid molecule inactive if it is connected through the γ carbon. This problem is more common when attaching the folic acid molecule to nanoparticles. In order to avoid this problem, the γ carbon is blocked using a small molecule such as N-hydroxy succinimide (NHS) [9]. However, when conjugating to a flat surface this doesn't seem to be a problem.

The cantilever sensor was coated with a 50 nm thick layer of titanium, applied using an e-beam deposition technique. The attachment of folic acid to the titanium surface requires an *organo-metallic* linker [21]. Such a linker has two ends, one which binds to an organic molecule and the other that binds to a metal. A more or less conventional method to conjugate organic molecules to a titanium or silicon surface is using a silane linker [22]. This method has been used to conjugate peptides and as a primer for dental and implant materials [2, 15, 16, 20, 26]. Silane reacts with the native oxide layer found on titanium surfaces. The protocol used for conjugating folic acid to the titanium surface is adapted from literature [5, 11, 26] and is outlined below. Modifications were made to ensure that the process is benign to the mechanically fragile cantilever sensors.

2 Materials and methods

The conjugation chemistry was developed on titanium rings which were suitable for conducting biochemical validation experiments. The wire for the titanium rings was provided *ex gratis* by Reactive Metals Studio, Clarkdale AZ. Other reagents and materials were obtained from Sigma-Aldrich, St. Louis, MO. The derivatization was carried out in several steps:

2.1 Pretreatment of the Ti surface

A 50 ml 1:1 v/v mixture of methanol and 37% HCl was prepared. The titanium surfaces were incubated in this solution for 30 min. They were rinsed in a beaker containing deionized water, changing the water 5 times. They were then immersed in concentrated sulfuric acid (H_2SO_4) for 15 min. Following that, the titanium surfaces were rinsed in deionized water followed by acetone and were oven dried under vacuum overnight. This procedure hydroxylates the native oxide layer on titanium surface.

2.2 Silanization of the hydroxylated layer

The silane used for this procedure was (3-aminopropyl)triethoxy silane (APTES) obtained from Sigma. The hydroxylated titanium surfaces were immersed for 12 h in a beaker containing 30 ml. of dry toluene to which 0.5 ml of 2.15 mmol APTES was added. The titanium rings/cantilevers were rinsed in chloroform, deionized water, acetone, methanol and again in acetone in succession. Finally they were removed to a glass petri dish and air-dried at room temperature.

2.3 Deployment of folic acid

An excess of folic acid (approximately 200 mg) was dissolved in warm (40–45°C) dimethyl sulfoxide (DMSO). To this mixture, 200 μl of pyridine and 160 μl of dicyclohexyl carbodiimide (DCC) were added. DCC functions as a heterobifunctional crosslinker between the amine group on the silane and the α carbon of folic acid. DCC is a waxy, solid substance at room temperature. It has a melting point of DCC is 37°C. In order to remove the required quantity from the container, it is necessary to melt the DCC before adding it to the DMSO. A pipette was used to remove 160 μl of DCC which solidifies inside the pipette tube instantly. Hence the necessity to warm DMSO to about 40°C to allow the DCC to melt before dissolving. The titanium surfaces were incubated in this solution for 3 h. They were rinsed in cold acetone, vacuum dried and stored in a glass dessicator.

3 Validation of the derivatization technique

An enzyme-ligated immunosorbent assay (ELISA) test was used to determine the presence and biological activity of the folic acid conjugated to the titanium surfaces. As mentioned before, the titanium rings were of a convenient size to fit inside the standard 96-well ELISA plate and were used for the biochemical tests. All titanium ring samples and the ELISA well plates were blocked in a 1% solution of bovine serum antigen (BSA) to reduce background signal due to non-specific binding. The primary antibody used was the mouse antibody to folic acid. A goat antibody to mouse linked to horseradish peroxidase (HRP) was used as the secondary antibody. HRP acts on a substrate, *o*-phenylenediamine dihydrochloride (OPD) to produce a yellow-brown color in proportion to the amount of secondary antibody present. The intensity of this color was read using a spectrophotometer operating at 490 nm wavelength. The results of the ELISA test are shown in Fig. 3. A negative control and controls for

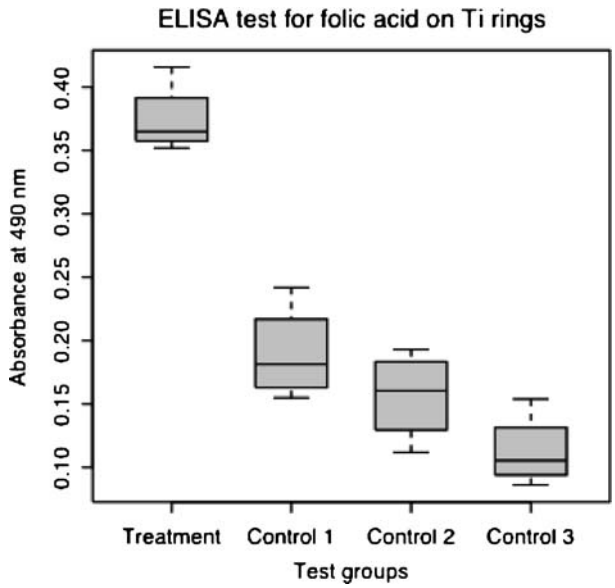

Fig. 3 ELISA test to detect presence of folic acid. Figure shows a box-whiskers plot representing the ELISA test for folic acid. Group 1 represents the signal due to folic acid. Control 1 is the negative control for when folic acid is absent, Control 2 is the non-specific binding of the secondary antibody when folic acid is present and Control 3 is the non-specific binding of the secondary antibody when folic acid is absent. The box-whisker plot shows the mean of the reading and the 25 and 75 percentile values with the horizontal lines and the extreme values seen in the data using the "whiskers". The average blank reading was 0.038 units. (not shown)

non-specific binding of primary and secondary antibodies was also placed in the validation procedure. The box and whiskers plot shows that the folic acid molecule on the titanium rings evokes a significantly stronger ELISA response that the control cases. It also shows that the relatively small folic acid molecule is visible to the folate binding protein (the primary antibody) even after being blocked with the bovine serum antigen (BSA).

4 Effect of derivatization on cantilever performance

The performance of a sensor can be usually described in terms of its sensitivity, selectivity and specificity. In case of the cantilever sensor, the sensitivity is a function of the structure and geometry of the transducer and has been previously modeled [1]. The selectivity and specificity are characteristics of the recognition layer, which in this case is the folic acid surface treatment on the sensor.

Selectivity is defined as the ability with which the sensor can detect the analyte when it is present. In case of the cantilever sensor, it is the proportion of the cases in which it shows a drop in frequency when exposed to cancer cells. Mathematically, it is the probability of getting a true positive result given that the disease is present. Specificity on the other hand is defined as the ability of the sensor to correctly identify the absence of the disease. For the cantilever sensor, this means it is the proportion of trials in which it accurately registers no frequency change when subject to non-cancerous cells. Thus mathematically, selectivity and specificity are defined as:

$$Selectivity = \frac{TP}{TP + FN} \tag{1}$$

$$Specificity = \frac{TN}{TN + FP} \tag{2}$$

where,
TP is the number of True Postives,
FP is the number of False Postives,
TN is the number of True Negatives and
FN is the number of False Negatives

In order to test the effect of the functional coating on the cantilever sensor, 50 cantilever structures were coated with titanium at Princeton University. Of these, 40 were successfully conjugated as per the protocol developed. The rest were damaged during the process. Simultaneously, KB cells (ATCC catalog number CCL-17) were cultured in the Davis Heart & Lung Research Center at The Ohio State University. The cells were cultured in folate replete, serum enriched, modified Eagle's medium. The KB cells were split into two flasks. One flask was treated with regular media while the cells in the other flask were cultured in folate deplete media, which leads to an overexpression of folate receptors on the KB cell surface [12, 24].

The initial resonant frequencies of the cantilever structures were measured. 25 of the 40 cantilevers were exposed to the KB cells for one hour. As a control, the remaining 15 cantilevers were exposed to cells in a suspension containing an excess of folic acid in order to encourage competitive binding. Folic acid from the media binds to the folate receptors making them temporarily unavailable to participate in binding on the sensor surface. The results of the cantilever binding experiments is shown in Fig. 4. In case where the cantilevers were exposed to cells having available folate receptors, 22 cases showed true positive results with frequency decreases. One case showed a false negative with no frequency change and two tips were damaged. In the cases where the cantilevers were exposed to cells having their folate receptors occupied competitively using folic acid, 7 false positive cases were seen. In 5 additional cases, frequency increases were seen. 3 cantilevers were damaged. From Equation 1 above, it is possible to compute the selectivity of the sensor, which is $22/23 \approx 95.6\%$. Since it is not easy to determine the number of true negatives, the specificity of the sensor cannot be computed.

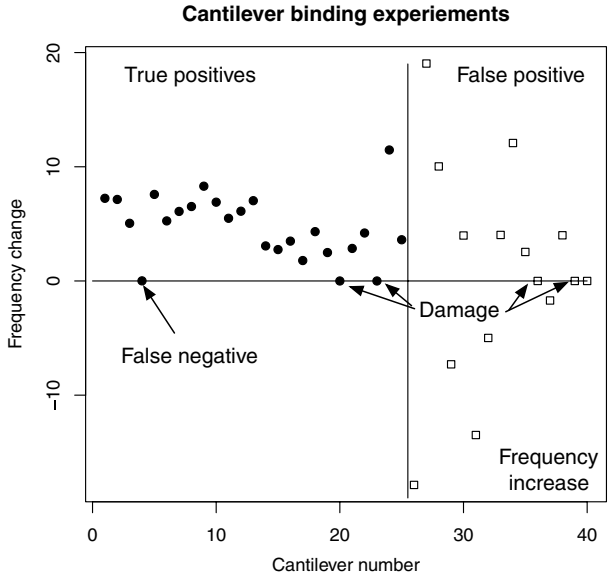

Fig. 4 Results of the cantilever binding experiments. Figure shows the change in frequency of the 40 cantilevers. Solid circles indicate tests on folate receptor positive cells. Open squares indicates tests where the receptor was competitively bound. Y axis shows the frequency change in KHz, with the positive axis showing the drop in frequency. Top left corner indicates true positives and top right corner indicates false positives. A single false negative reading was seen. 5 cantilevers were damaged and yielded no results. 5 cases showed frequency increases

5 Interpretation of results and discussion

From the results noted above, the positive and consistent drops in frequency of the cantilever subjected to cancer cells suggests its potential in cancer detection. The folic acid-based cantilever sensor is more selective for cancer cells with an overexpression of the folate receptor than those with unavailable receptors. This makes is a viable candidate in the development of devices that utilize folic acid as the recognition layer for cancers that show overexpression of the folate receptor. Such cancers include nasopharyngeal and ovarian carcinomas.

Although the high selectivity of the sensor shows promise in the analysis of suspect tumor tissue, the number of false positives are clearly an issue. A possible hypothesis is that once the cells are removed from the folate rich medium into the testing chamber, the folate receptor may be getting recycled rapidly after the internalization of the folic acid by the reduced folate carrier (RFC), making it available again for cell attachment. The rate of folate internalization by the (RFC) is not currently known. This biological process is worth investigating as it has implications from a drug-delivery standpoint.

The false positives make for a reduction in the specificity of the sensor. Possible solutions to this problem include developing an array of selective sensors for multiple parameters and developing a decision support system for diagnosis and prognosis. Another solution is to redesign the recognition layer to prevent non-specific cell attachment.

The frequency increases were an unexpected phenomenon, likely caused due to protein deposits on the cantilever surface. Changes in surfaces stresses on the cantilever are likely to change its resonance properties. It is possible that the surface stresses leading to increase in the resonance frequency are a competing phenomenon that leads to smaller drops in resonance frequency than expected. The effects of these surface stresses on the mechanical properties of the cantilever transducer may be elucidated by performing experiments on the static deflection of cantilevers. The design of such experiments is in progress.

6 Conclusion

The cantilever-based system is certainly a viable *platform technology* for biosensor applications ranging from small molecules to cells. However, in view of the biological complexity of disease detection and reliable diagnosis the cantilever sensor needs to be complimented with other sensing modalities as well as computational tools from bioinformatics. The sensor, along with other allied technologies and the quantitative framework provided by bioinformatics and systems biology, can potentially bridge the gap between biomarker research and pathological inference of disease.

The vision for a complete, reliable and efficient diagnostic system consists of an array of different sensors, not necessarily all MEMS-based, specially tuned to measure different aspects of the disease or phenomenon of interest. The results for all these analytes should be viewed and interpreted jointly in a systems biology-based framework in order to impart an insight into the systematic phenomena relevant to a disease.

Finally, MEMS devices, in general, would be truly successful if it is, by virtue of its design and performance, adopted by the medical research community as an enabling and empowering research tool in their ever-deepening study of biology, and by the engineering community to bootstrap itself into developing the next generation of biomedical technology.

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